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(54) Title: THE USE OF EXCITATORY AMINO ACID TRANSPORTER INHIBITORS TO REDUCE CNS WHITE MATTER INJURY

(57) Abstract

This invention provides a method of reducing the damaging effect of CNS white matter injury (including traumatic CNS injury, anoxia, and ischemia) to mammalian CNS tissue, particularly spinal cord tissue, by *in vivo* treatment thereof with inhibitors of excitatory amino acid transporters, such as the Na^+-dependent glutamate transporter and pharmaceutical compositions comprising said inhibitors.

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THE USE OF EXCITATORY AMINO ACID TRANSPORTER INHIBITORS TO REDUCE CNS WHITE MATTER INJURY

FIELD OF THE INVENTION

This invention relates to a CNS injury.

BACKGROUND TO THE INVENTION

The central nervous system consists of gray matter and white matter. Gray matter contains the cell bodies of neurons, embedded in a neuropil made up predominantly of delicate neuronal and glial processes. White matter, consists mainly of long processes of neurons, the majority being surrounded by myelin sheaths, and nerve cell bodies are lacking. Both gray and white matter contain large numbers of neuroglial cells and a network of blood capillaries. In some parts of the central nervous system, notably the brain stem (medulla, pons, and midbrain), there are regions that contain both nerve cell bodies and numerous myelinated fibers. These regions are therefore an admixture of gray matter and white matter.

There are a number of conditions that result in disruption of white matter integrity and function in the CNS. In addition to traumatic CNS injury, pathological states that involve disruption of white matter are: anoxia, ischemia, and demyelination. Stroke is a very common condition affecting both gray and white matter structures (Pantoni, *et al.*, *Stroke* (1996) 27:1641-1646). The mode of injury of white matter fibers includes Ca^{2+} overload mainly through reverse operation of the Na^{+}-Ca^{2+} exchanger (for a review see: Stys, *J. Cereb. Blood Flow Metab.* (1998) 18:2-25). More recent data implicate a component of glutamate excitotoxicity in anoxic white matter. Blocking), α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors is significantly neuroprotective against anoxic injury in isolated spinal cord white matter (Li & Stys, *J. Neurotrauma* (1998)15(10):880), and broad spectrum inhibition of glutamate receptors with kynurenic acid protects optic nerves (representative CNS white matter tracts) against *in vitro* anoxia as well.

Traumatic brain injury is known to cause significant non-disruptive axonal injury that contributes to the neuropsychological deficits frequently encountered in this condition. While the precise mechanisms of such axonal injury are not known, injury cascades seen in anoxic/ischemic and traumatic spinal cord injury are likely to contribute to white matter damage following head trauma as well (Maxwell, et al., *J. Neurotrauma* (1997) 14:419-440; Obrenovitch & Urenjak, *J. Neurotrauma* (1997) 14:677-698).

Acute, traumatic spinal cord injury (SCI) is a devastating clinical condition for which current treatment is only modestly effective. It usually results in lifelong disability for the patient and its effect is enormous in terms of the psychological, social, and financial costs to the patient, the family and society. The neurological deficits resulting from SCI are due primarily to damage to the nerve fibres that carry messages up and down the spinal cord.

Despite the clinical diagnosis of "neurologically complete SCI", the spinal cord itself is rarely transected, as demonstrated by routine post-mortem histopathological investigations that have characterized the anatomical integrity of the lesion site. These observations have been corroborated by more recent noninvasive magnetic resonance imaging techniques, which have correlated changes in spinal cord tissue in the living patient with the gross clinical histopathology obtained post-mortem.

There is increasing clinical and experimental evidence for significant preservation of descending tracts in neurologically complete SCI. In fact, use of the potassium channel blocker, 4-aminopyridine, in chronic spinal-injured cats and more recently in patients, provides strong evidence for the persistence of anatomically intact but physiologically dysfunctional descending supraspinal pathways. These drug studies in humans and in animals suggest that even though these remaining intact nerve fibres are dysfunctional, they may be induced to regain some physiologically significant function with the appropriate pharmacological intervention. More importantly, increasing the survival of spinal cord axons following the acute injury using

pharmacological intervention as proposed here, may significantly improve clinical outcome; even a small number of remaining functional fibers can support significant clinical function.

Identification of the factors or mechanisms that cause nerve fiber injury, particularly during the early post-injury period, could enable specific drug therapies to be targeted to maximize neurologic recovery. A large body of work exists dealing with the pathophysiology of gray matter anoxic and ischemic injury (for reviews see Seisjö, B.K., (1986) *Eur Neurol* 25:45-56; Choi, D.W., (1990) *J. Neurosci* 10:2493-2501; Haddad, G.G. and Jiang, C., (1993) *Prog Neurobiol* 40:277-318; Seisjö, B.K. and Wieloch, T., *Cellular and Molecular Mechanisms of Ischemic Brain Drainage* (New York: Raven Press, 1996), pp.527). An emerging theme implicates cellular overload of Ca^{2+}, occurring largely through glutamate-gated receptors and possibly voltage-gated Ca^{2+} channels, cell swelling as a result of excessive Na^+ and Cl^- influx, free radical production, and delayed apoptotic neuronal death. In contrast, much less is known about the fundamental mechanisms of anoxic and ischemic injury to CNS myelinated axons, despite the fact that white matter has been shown to be very vulnerable to this type of injury (Follis, F., *et al.*, (1993) *J Cereb Blood Flow Metab* 13:170-178; Kochhar, A., *et al.*, (1991a) *Brain Res 542:141-146*; Kochhar, A., *et al.*, (1991b) *J Neurotrauma* 8:175-186; von Euler, M., *et al.*, (1994) *Exp Neurol* 129:163-168).

Transporter proteins play a particularly important role in uptake of extracellular amino acids in the vertebrate brain (see Nicholls & Attwell, 1990, *TiPS* 11: 462-468). Amino acids that function as neurotransmitters must be scavenged from the synaptic cleft between neurons to enable continuous repetitive synaptic transmission. More importantly, it has been found that high extracellular concentrations of certain amino acids (including glutamate and cysteine) can cause neuronal cell death. High extracellular amino acid concentrations are associated with a number of pathological conditions, including ischemia, anoxia and hypoglycemia, as well as chronic illnesses such as Huntington's disease, Parkinson's disease, Alzheimer's disease, epilepsy and amyotrophic lateral sclerosis (see Pines *et al.*, 1992, *Nature* 360: 464-467).

Glutamate is one example of such an amino acid. When present in excess (>about 300 μM; Bouvier *et al.*, 1992, *Nature* 360: 471-474; Nicholls & Attwell, ibid.; >5 μM for 5 min.; Choi *et al.*, 1987, *J. Neurosci.* 7: 357-358), extracellular glutamate causes neuronal cell death. Glutamate transporters play a pivotal role in maintaining non-toxic extracellular concentrations of glutamate in the brain.

During anoxic conditions (such as occur during ischemia), the amount of extracellular glutamate in the brain rises dramatically. This is in part due to the fact that, under anoxic conditions, glutamate transporters work in reverse, thereby increasing rather than decreasing the amount of extracellular glutamate found in the brain. The resultantly high extracellular concentration of glutamate causes neuron death, with extremely deleterious consequences for motor and other brain functions.

It has been considered that there are at least three glutamate pools that can contribute to glutamate release during CNS insults. One is Ca^{2+} independent reversal of the glutamate transporter (Nicholls and Atwell, 1990; Szatkowski *et al.*, 1990, *Nature* 348:443-446; Attwell *et al.*, 1993, *Neuron* 11:401-407). Another mechanism seen in primary astrocyte cultures is a swelling-induced Ca^{2+} independent release (Kimelberg *et al.*, 1990, *J Neurosci* 10:1583-1591). It has been proposed that inhibition of uptake and/or reversal of the glutamate transporter can occur when the electrochemical gradients for Na^+ and K^+ are disrupted during CNS insults; these effects can contribute significantly to the increased $[glu]_0$ seen during pathological states (Hansen, 1985, *Physiol Rev* 65:101-138; Ikeda *et al.*, 1991, *J Biol Chem* 266:12058-12066; Attwell *et al.*, 1993; Wahl *et al.*, 1994, *J Neurochem* 63:1003-1011).

Ordinarily there is tight regulation of extracellular glutamate levels, which are normally measured to be approximately 1-2 μM (Erecinska and Silver, 1990, *Prog Neurobiol* 35:245-296). High-affinity Na^+ dependent glutamate transporters are thought to be primarily responsible for maintaining this low extracellular glutamate concentration and are present on both neurons and astrocytes. There are now known to be at least three different subtypes of glutamate transporters

(GLAST-1, GLT-1 and EEAC1) in the rat, as well as an EAAT4 isoform in human cerebellum (Pines *et al.*, 1992; Kanai and Hediger, 1992, *Nature* 360:467-471; Storck *et al.*, 1992, *Proc Natl Acad Sci USA* 89(22):10955-10959; Wadiche *et al.*, 1995, *Neuron* 14:1019-1027). Recent work has suggested that GLAST-1 and GLT-1 are primarily responsible for maintaining low $[glu-]_0$ (Nicholls and Attwell, 1990; Rothestein *et al.*, 1994, *Neuron* 13:713-725).

GLAST-1 and GLT-1, appear to be specific to brain and are expressed predominantly in glia. GLAST-1 mRNA is expressed diffusely throughout the cerebrum, but is restricted to the Purkinje cell layer of cerebellar cortex. EAAC-1 is expressed abundantly in brain, intestine, and kidney and at lower levels in liver and heart. Within brain, EAAC-1 mRNA is found predominantly in neurons, especially in certain neuronal subsets of hippocampus, cerebellar cortex, and cerebral cortex. These three transporters have about 50 percent sequence similarity and an inferred topology of 6 to 10 transmembrane segments. They all energize amino acid uptake by Na^+ symport coupled to K^+ antiport and are Cl^-–independent. While they are all specific for glutamate and aspartate, the details of glutamate transport may vary among them.

Many studies have been performed over the years on the effects of anoxia and ischemia in peripheral axons (for a review see Stys, P.K., *et al.*, (1995) in Waxman, S.G., *et al.*, eds., *The Axon: Structure, Function and Pathophysiology* (New York: Oxford University Press, 1995), pp. 462-479). Despite the strong similarity in structure and function between central and peripheral fibers, their responses to energy failure are quite different, and therefore findings in the peripheral nervous system cannot be extrapolated to central axons.

For example, in the case of hypoxia, an elegant study by Utzschneider, D.A., *et al.*, ((1991) *Brain Res* 551:136-141), emphasized the dramatically different effects of hypoxia on central (dorsal root ganglion cell processes in spinal dorsal columns) versus peripheral (dorsal roots) axons. The central component is very sensitive to hypoxia, whereas the peripheral projections originating from the same cell are completely unaffected by a 30 min exposure. Even more

striking is the observation that although longer hypoxic exposures result in significant deregulation of elemental content (including large accumulations of Ca^{2+}) in the axoplasm of peripheral myelinated fibers, reoxygenation promotes complete recovery (Lehning, E.J., *et al.*, (1966b) *Brain Res* 715:189-196); this is in stark contrast to central axons, in that not only does reoxygenation fail to correct the pathologic translocation of ions caused by the hypoxic insult, but many axons continue to deteriorate, accumulating more Ca^{2+} and presumably suffering more damage (Stys, P.K. and LoPachin, R.M., (1996) *Neuroscience* 73:1081-1090).

Figure 1 summarizes the current understanding regarding the unique as well as overlapping events responsible for gray and white matter anoxic and ischemic injury (Stys, PK., (1998) *J. Cereb. Blood Flow Metab* 18:2-25). Both gray and white matter are vulnerable to interruption of energy supply, which leads to impairment of ion transport and collapse of transmembrane ion gradients. Glutamate-gated channels play a central role in gray matter pathophysiology, allowing Na^+ and Ca^{2+} entry, whereas Na^+ channels are the main Na^+ influx pathway in myelinated axons. The resultant cellular Na^+ overload and membrane depolarization may further activate certain subtypes of voltage-sensitive Ca^{2+} channels (VSCC) in gray matter, while promoting Ca^{2+} overload largely through reverse Na^+-Ca^{2+} exchange in white matter. Downstream injury mechanisms likely then converge, involving excess activation of Ca^{2+}-dependent biochemical pathways, generation of free radicals, and mitochondrial injury, culminating in cell death. Optimal protection of the CNS as a whole will therefore require combination therapy aimed at unique steps in gray and white matter regions, or intervention at common points in the injury cascades.

There is substantial evidence in the literature that following the initial mechanical impact of traumatic CNS and spinal cord injuries, sequential and progressive tissue damage occurs at the injury site. These observations have given rise to the secondary injury hypothesis which implicates a cascade of neuropathological mechanisms in the post-traumatic destruction of spinal cord tissue. Included in the list of secondary injury mechanisms are post-traumatic ischemia and the release of excitotoxic amino acids.

Death of neurons following traumatic or ischemic disorders has been related to excess intracellular calcium which occurs, for example through excessive activation of post-synaptic glutamate receptors (Choi, D.W. and Rothman, S.M., (1990) *Ann Rev Neurol* 13:171-182; Young, W. (1992) *J Neurotrauma* 9(suppl):s9-s25). Considerably less is known about glial cell death, although electrophysiological and pharmacological studies indicate that glial cells probably do not have the same complement of glutamate receptors as do neurons.

A need therefore remains for an effective method of reducing the impact of CNS white matter injury.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide the use of excitatory amino acid transporter inhibitors to protect against CNS white matter injury. In accordance with an aspect of the present invention there is provided an *in vivo* method of reducing the deleterious effect of traumatic CNS tissue injury by applying to said tissue a therapeutically effective amount of an inhibitor to Na^+ dependent glutamate transporter.

It is an object of the present invention to provide in its broadest aspect a method of reducing the damaging effect of traumatic CNS injury, anoxia, and/or ischemia to mammalian CNS white matter.

It is a further object of the invention to provide pharmaceutical compositions for use in treating mammals to reduce the damaging effect of traumatic CNS injury, anoxia, and/or ischemia to mammalian CNS white matter.

The present invention is based on a determination of a neuroprotective effect against acute, anoxic or traumatic CNS and spinal cord injury by the manipulation of Na^+-dependent glutamate

transporter. This determination is applicable to all myelinated nerve tracts in the CNS and further addresses the possibility that neurotoxicity results from the *accumulation* of glutamate molecules in the external environment of myelinated axons.

Accordingly, the invention provides in one aspect an *in vivo* method of reducing the deleterious effect of traumatic CNS injury, anoxia, and/or ischemia to mammalian CNS white matter by applying to said tissue a therapeutically effective amount of an inhibitor to a Na^+-dependent glutamate transporter.

Preferred compounds for use in reducing the deletrious effects of traumatic CNS injury, anoxia, and/or ischemia to mammalian CNS white matter are dihydrokainate (DKA), L-trans-2,4-pyrrolidine dicarboxylate (L-trans-PDC), L-anti-endo-3,4-methanopyrrolidine dicarboxylate and its isomers, DL-threo-beta-hydroxyaspartic acid, L-cysteic acid, L-cysteine sulfinic acid, (2S, 1'S, 2'R)-2-(carboxycyclopropyl)glycine, and closely related analogs.

In a further aspect the invention provides a therapeutic composition for reducing the deleterious effect of *in vivo* traumatic CNS tissue injury comprising a therapeutically effective amount of an inhibitor to a Na^+-dependent glutamate transporter and a pharmaceutically acceptable carrier, diluent or adjuvant therefore.

BRIEF DESCRIPTION OF THE FIGURES

Table 1 present results of anoxia studies using: 1A) GYKI 52466 and anoxia - anoxia applied from 60 to 120 min; 1B) DKA & PDC and anoxia - anoxia applied from 60 to 120 min.

Table 2 presents the results of spinal cord injury (SCI) studies: 2A) GYKI 52466 & SCI: SCI applied briefly right after 60 min mark; 2B) PDC & SCI: SCI applied briefly right after 60 min mark.

Figure 1 presents a comparison of events in gray and white matter leading to anoxic / ischemic injury of CNS tissue.

Figure 2 presents a demonstration of the protective effects against anoxia of a selective AMPA receptor antagonist, GYKI 52466. GYKI52466 was applied beginning 1 hour before *in vitro* anoxia of spinal dorsal column slice. Normalized compound action potential (CAP) amplitudes are plotted against time, in the absence and presence of GYKI52466 (panel A) (* $P < 0.05$). Panel B shows representative CAP tracings. These results indicate that glutamate, acting largely via AMPA receptors, participated in anoxic spinal dorsal column injury. (From Li, Mealing, Morley and Stys, *J. Neurosci.*, 1999).

Figure 3 presents a demonstration of protective effects of GYKI 52466 in *in vitro* traumatic spinal cord injury. In particular, these results demonstrate that glutamate, acting largely via AMPA receptors, participates in traumatic spinal dorsal column injury. GYKI 52466, a selective AMPA antagonist was applied beginning 1 hour before *in vitro* clip compression of spinal dorsal column slice, and demonstrated a highly protective effect against SCI (panel A) (* $P < 0.05$). Representative CAP tracings are shown in panel B. (From Li, Mealing, Morley and Stys, *J. Neurosci.*, 1999).

Figure 4 shows protective effects of Na^+-dependent glutamate transporter inhibitors: dihydrokainate (DHK) or L-trans-PDC (PDC). In separate preparations, these inhibitors were applied beginning 1 hour before *in vitro* anoxia of spinal dorsal column slice. Bar graph shows significant protective effect measured by compound action potential (CAP) amplitudes. These results indicate highly protective effect against anoxia. Panel B shows fluorescent measurements of intracellular glutamate in axons and oligodendrocytes. Anoxia causes significant depletion of this excitatory neurotransmitter from both compartments, which is completely reversed by inhibition of Na^+-dependent glutamate transport. These results directly demonstrate that glutamate is released by reverse transport during anoxia, and that inhibition of reverse transport is significantly neuroprotective (* $P < 0.05$). (From Li, Mealing, Morley and Stys, J. Neurosci.,

1999).

Figure 5 shows the protective effects of an exemplary Na^+-dependent glutamate transporter inhibitor, L-trans-PDC, in a model of traumatic spinal cord injury. L-trans-PDC was applied 1 hour before *invitro* SCI of spinal dorsal column slice induced by clip compression. These results show a highly protective effect against SCI and indicate that glutamate is likely released by reverse transport during SCI (* $P < 0.05$).

DETAILED DESCRIPTION OF THE INVENTION

This invention is based on pharmacological agents aimed at inhibiting (completely or incompletely) reverse excitatory amino acid transport (such as glutamate transport) that can be used as neuroprotectants against traumatic, ischemic and/or anoxic CNS white matter injury.

Some preferred compounds for use in reducing the deletrious effects of traumatic CNS injury, anoxia, and/or ischemia to mammalian CNS white matter are: dihydrokainate (DKA), L-trans-2,4-pyrrolidine dicarboxylate (L-trans-PDC), L-anti-endo-3,4-methanopyrrolidine dicarboxylate and its isomers (Bridges *et al., Neurosci Lett.* 174:193-7); DL-threo-beta-hydroxyaspartic acid acid (Arriza, *et al.,* 1994, *J. Neurosci.*, 14:5559-69); L-cysteic acid (Arriza, *et al.,* 1994, *J. Neurosci.*, 14:5559-69); L-cysteine sulfinic acid (Arriza, *et al.,* 1994, *J. Neurosci.*, 14:5559-69); (2S, 1'S, 2'R)-2-(carboxycyclopropyl) glycine (Yamashita, et al., 1995, Eur J. Pharmacol 289:387-90); and closely related analogs. Another preferred compound is DL-TBOA (Shimamoto, K. et al., (1998) *Mol. Pharmacol.*).

The term, Na^+dependent glutamate transporter, means any protein or isoform determined to be a member of the family of the Na^+ dependent transporters of glutamate. Some of these transporters may be known to also transport other amino acids or neurotransmitters, so the transport activity need not be exclusive to glutamate. Common examples of transporters are the GLAST isoforms, the GLT isoforms and the EAAC isoforms. Newly identified Na^+ dependent

transporters of glutamate that function in the manner described herein, also fall within the scope of this invention.

The term, inhibition, means complete or incomplete inhibition of Na+ dependent transporter activity.

Compounds that inhibit (completely or incompletely) Na^+ dependent glutamate transport are used in the methods of this invention. A series of qualifying criteria are provided herein to determine whether a compound can be used in the method of this invention. One skilled in the art would appreciate that compounds that can be used in the method of this invention would demonstrate the desired activity in one or more of the tests that establish qualifying criteria. It would also be appreciated that new techniques developed to determine the same activity of inhibiting excitatory amino acid transport could also be used as such a test.

Tests to Establish Qualifying Criteria

There are a number of assays known in the art that are useful for determining whether a compound inhibits Na^+ dependent transport activity.

Test No. 1: *In Vitro* Anoxia Assay

This assay determines the ability of the test compound to block the reduction of electrical activity following anoxia. In one example, 60 min of N_2/CO_2 exposure in slices of rat dorsal column is used as an *in vitro* model of anoxia. Functional integrity is measured electrophysiologically by recording propagated extracellular compound action potentials. The tissue is rendered anoxic by N_2/CO_2 exposure followed by re-oxygenation. Post-anoxic CAPs are measured at 1 and 2 hrs of re-oxygenation and compared to pre-anoxic responses thus yielding a percent recovery. Glutamate transport blockers are typically applied beginning 30-60 min before the start of anoxia. Improvement in post-anoxic recovery compared to drug-free

controls is an indicator of protective efficacy of putative glutamate transport inhibitors. In addition, a direct measure of glutamate release from axons and glia is performed by quantitative immunohistochemistry for glutamate. This allows a direct relative measure of cellular glutamate changes during anoxia in the absence or presence of glutamate transport inhibitors (Li, Mealing, Morley and Stys, *J. Neurosci.*, 1999). A reduction of cellular glutamate loss during anoxia in the presence of inhibitor is a strong indication of said agent's ability to reduce toxic glutamate release.

Test No. 2: *In Vitro* Mechanical Injury Assay

This assay determines the ability of the test compound to block the reduction of electrical activity following *in vitro* mechanical injury. In one example 15 sec. of clip compression @ 2 grams in slices of rat dorsal column is used as an *in vitro* model of mechanical injury. As in Test No. 1, the compound action potential is used as a measure of functional integrity, and post-traumatic compared to pre-traumatic responses give an indication of recovery after *in vitro* clip compression. Glutamate transport blockers are typically applied beginning 30-60 min before compression. Improvement in CAP amplitude in the presence of glutamate transport inhibitor reflects this agent's neuroprotective activity as it relates to reduced glutamate transporter activity and consequently diminished release.

Test No. 3: Measurement of Labeled Glutamate or Aspartate Uptake

Compounds can be screened to identify those which specifically interact with a Na^+ dependent glutamate transporter and decrease its ability to take up a neurotransmitter, e.g., an antagonist/inhibitor. One method comprises transforming host cells with a vector encoding a Na^+ dependent glutamate transporter, such that a transporter polypeptide is expressed in that host, incubating the host cells with glutamate, aspartate or analog thereof which has been labeled by a detectable marker sequence (e.g., radiolabel or a non-isotopic label such as biotin) and the potential compound and determining whether translocation of the neurotransmitter into the cell is

either inhibited or increased. By measuring the amount of neurotransmitter inside the cell, one skilled in the art could determine whether the compound is an effective antagonist.

Test No. 4: Measurement of Labeled D-Aspartate Efflux

Primary astrocyte cultures can be used to assay the efflux of preloaded tritiated D-aspartate in the presence of the test compound. [^{3}H]-D-aspartate is used as a nonmetabolizable marker for the intracellular glutamate and aspartate pools. Both of these amino acids are transported on the same carrier protein and label the nonvesicular pool of excitatory amino acids (Erecinska and Silver, *Prog Neurobiol* (1990) 35:245-296; Barbour *et al.*, *J Physiol (Lond)* (1993) 466:573-597.

In one example of this test, astrocytes grown on coverslips are incubated overnight in 2.5 ml of MEM containing 10% horse serum, together with 4 µCi/ml of [^{3}H]-D-aspartate (1 mCi/ml; specific activity 86.4 mCi/mg aspartate). Optionally, 8 µCi/ml $Na_2{}^{51}CrO_4$ is added to the incubation medium (1 mCi/ml; specific activity, 50mCi/mg Cr). The appearance of ^{51} Cr in the perfusate during release experiments can be used to determine whether an increase in [^{3}H]-D-aspartate release is a result of cell detachment or lysis (Kimelberg et al., Brain Res (1993) 622:237-242. The loaded coverslips are inserted into a Lucite perfusion chamber with a cut out depression in the bottom for the 18 x 18 mm glass coverslips. The chamber has a screw top and when screwed down leaves a space above the cells of around 100 µm. This perfusion chamber is well suited for measuring the release of [^{3}H]-D-aspartate from astrocytes in response to KCl buffer because the volume in the chamber is relatively small (18 x 18 x 0.1 mm = 32.4 µl). This chamber allows a complete change of the perfusing buffer within 2 min, as determined by removal of a trypan blue solution.

The cells are perfused with HEPES-buffered solution consisting of 140 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM (+)D-glucose, 25 mM HEPES. NaOH (10 N) was used to pH the buffers to 7.4. Increased KCl buffers were made by replacing NaCl with KCl. The osmolarity of all buffers are measured by a freezing point osmometer

(Advanced Instruments, Neeham Heights, MA); the osmolarities are 285-290 mOsm. Sucrose is added to make any adjustments in osmolarity to exactly 290.

The Lucite chamber and a fraction collector are placed in an incubator set at 37°C, and the perfusate is collected in 1 min intervals. At the end of the experiment, the cells are digested off the coverslip with 1N NaOH. The radioactivity is counted using a liquid scintillation analyzer. Percent fractional release for each point is calculated by summing the radioactive counts from the end time point to the beginning of each minute plus the radioactivity left in the cell digest and dividing the dpms released in each minute by these summed dpms.

One skilled in the art would appreciate that the multitude of pharmacological effects of the candidate compounds could have to be taken into account in determining usefulness and efficacy for *in vivo* utility.

Means of delivering the Na^+-dependent glutamate transport inhibitors to the site of injury are known in the art. Administration may be by injection, including intravenous injections. Catheters are another preferred mode of administration, potentially into the intrathecal space.

Formulations may be any that are appropriate to the route of administration, and will be apparent to those skilled in the art. The formulations may contain a suitable carrier, such as saline, and may also comprise bulking agents, other medicinal preparations, adjuvants and any other suitable pharmaceutical ingredients.

EXAMPLES

EXAMPLE I: Demonstration That Glutamate Applied Exogenously is Injurious to Axons

An *in vitro* model of spinal white matter anoxia and trauma demonstrates that glutamate applied

exogenously is highly injurious, probably acting through AMPA/kainate receptor subtypes. For example, using an electrophysiological assay, the electrical response is attenuated to about 50% of control amplitude after 120 minutes of glutamate exposure; 60 min of wash did not reverse this injury. In contrast, glutamate applied with antagonist such as NBQX or kynurenic acid results in more than 80% activity after a similar exposure

EXAMPLE II: Demonstration of glutamate release via reverse of Na^+-dependent transporters.

This study demonstrates the effect of glutamate on the compound action potentials (CAP) recorded from isolated dorsal column segments *in vitro* at 37°C. CAP amplitude decreased significantly (eg. to 54% of control after 120 min) after perfusion with 1 mM glutamate for 90 - 180 minutes in comparison with the time zero or time-matched controls in glutamate-free perfusate ($P<0.01$ The immunocytochemical distribution of high affinity Na^+-dependent glutamate transporters, GLT1, GLAST and EAAC1, in dorsal columns was studied with confocal microscopy. It was found that GLT1 and EAAC1 displayed similar patterns and were seen on the outer myelin sheath and at the nodal gap. Faint axoplasmic signal was also detected, accentuated at the nodal constriction. Although GLAST staining was found in similar regions, in contrast to GLT-1 and EAAC1, the myelin sheath appeared to be stained throughout its entire thickness with GLAST label.

Therefore it is concluded that *in vitro* application of glutamate results in the functional impairment of myelinated axons in dorsal spinal cord. Under pathological conditions such as trauma or ischemia, glutamate may be released in a non-vesicular fashion from axons and/or glia via reverse operation of Na^+-dependent transporters, leading to damage to nearby myelin, supporting glia and possibly the axon cylinder itself.

EXAMPLE III: Demonstration of the Role of Endogenous Glutamate in Two Injury Paradigms

Two injury paradigms were used to demonstrate the role of endogenous glutamate: *in vitro* anoxia (60 min of N_2/CO_2 exposure) and *in vitro* mechanical injury (15 sec. of clip compression

@ 2 grams), in slices of rat dorsal column. Both paradigms result in a reduction of electrical activity to about 25% (anoxia) and 35% (trauma) of control. Applying the selective AMPA receptor blocker GYKI 52466 allowed recovery to more than 55% after anoxia vs. 25% control (Figure 2), and to 65% after trauma vs. 35% control (Figure 3). Endogenous glutamate is therefore released causing significant injury, acting largely (although not necessarily exclusively) at the AMPA subtype of glutamate receptor.

EXAMPLE IV: Demonstration that Glutamate is Released from the Axon Cylinder and/or Glia

The axon cylinder is known to possess high concentrations of glutamate in the axoplasm. Both anoxia (LoPachin & Stys (1995) *J. Neurosci.*, 15:6735-6746) and mechanical trauma (Blight & LoPachin, *Soc. Neurosci Abstr* (1998)24:251) cause ionic perturbations that would lead to glutamate release via reverse Na^+-glutamate transporters which are present in these axons. Direct measurements (see Figure 4B) show that the source of glutamate is the axon cylinder and glia. In particular, these studies demonstrate that the release mechanism is reversal of the Na^+-dependent glutamate transporter that normally takes up glutamate into the cell. Under abnormal conditions, with high internal Na^+ and membrane depolarization, transport is reversed and glutamate can be released instead, as has been described in gray matter ischemia. This has never been demonstrated in white matter anoxia or trauma.

Spinal cord slices *in vitro* were treated with Na^+-dependent glutamate transport blockers (dihydrokainate or L-trans-PDC). As demonstrated in Figure 4, both were highly neuroprotective against anoxia (dihydrokainate: 65% and L-trans-PDC; 75%, vs. 25% without blockers), and Figure 5 shows the results in trauma (L-trans-PDC; 70% vs. 35% without inhibitor).

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

1A

Amplitude	means Ctrl no drug	means GYKI	SD Ctrl	SD GYKI
0m	100	100	0	0
60m	100	90.4130952	0	10.2149852
120m				
180m	26.6663796	56.0488095	4.48965603	16.527948
240m	25.2628337	56.8630952	6.62886587	13.1974253

1B

Amplitude	means Ctrl	means DKA	means PDC	SD Ctrl	SD DKA	SD PDC
0m	100	100	100	0	0	0
60m	100	99.6001536	94.6545216	0	13.6311537	15.3966918
120m						
180m	26.6663796	64.1344086	69.3066841	4.48965603	12.082054	12.604088
240m	25.2628337	60.5167435	76.5644823	6.62886587	12.231339	12.234225

TABLE 1

2A

Amplitude	means Ctrl	means GYKI	SD Ctrl	SD GYKI
0m	100	100	0	0
60m	100	100.921335	0	16.9880136
90m	37.0213086	48.3059757	13.1046774	16.9843585
120m	32.6292661	64.5894958	11.6263896	16.4124141
180m	35.2792219	65.6344071	14.2314234	14.5902904

2B

Amplitude	means Ctrl	means PDC	SD Ctrl	SD PDC
0m	100	100	0	0
60m	100	96.726215	0	16.8568686
90m	37.0213086	46.8528719	13.1046774	14.3666811
120m	32.6292661	63.024595	11.6263896	13.3041156
180m	35.2792219	70.6212077	14.2314234	7.62929473

TABLE 2

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An *in vivo* method of reducing the damaging effect of CNS white matter injury to mammalian CNS tissue by applying to said tissue a therapeutically effective amount of an inhibitor to an excitatory amino acid transporter.

2. A method as in claim 1, wherein said injury is traumatic CNS injury.

3. A method as in claim 1, wherein said injury is anoxia.

4. A method as in claim 1, wherein said injury is ischemia.

5. A method as in claim 1, wherein the excitatory amino acid transporter is a Na^+-dependent glutamate transporter.

6. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is L-trans-PDC.

7. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is dihydrokainate.

8. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is L-trans-2,4-pyrrolidine dicarboxylate.

9. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is L-anti-endo-3,4-methanopyrrolidine dicarboxylate.

10. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is an isomer of L-anti-endo-3,4-methanopyrrolidine dicarboxylate.

11. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is DL-threo-beta-hydroxyaspartic acid.

12. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is L-cysteic acid

13. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is L-cysteine sulfinic acid.

14. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is (2S, 1'S, 2'R)-2-(carboxycyclopropyl)glycine, and closely related analogs.

15. A method as claimed in claim 1 wherein said inhibitor to Na^+-dependent glutamate transporter is DL-TBOA.

16. A method as claimed in claim 1 wherein said CNS tissue injury is a spinal cord injury.

17. A method as claimed in claim 5 wherein said inhibitor comprises one or more compounds, in combination, selected from the list comprising: L-trans-PDC; dihydrokainate; L-trans-2,4-pyrrolidine dicarboxylate; L-anti-endo-3,4-methanopyrrolidine dicarboxylate; an isomer of L-anti-endo-3,4-methanopyrrolidine dicarboxylate; DL-threo-beta-hydroxyaspartic acid; L-cysteic acid; L-cysteine sulfinic acid; (2S, 1'S, 2'R)-2-(carboxycyclopropyl)glycine; DL-TBOA; and closely related analogs thereof.

18. A kit for reducing the damaging effect of CNS white matter injury to mammalian CNS tissue comprising: a syringe for intravenous injection and a formulation comprising is one or more compounds selected from the list comprising: L-trans-PDC; dihydrokainate; L-trans-2,4-pyrrolidine dicarboxylate; L-anti-endo-3,4-methanopyrrolidine dicarboxylate; an isomer of L-anti-endo-3,4-methanopyrrolidine dicarboxylate; DL-threo-beta- hydroxyaspartic acid; L-cysteic acid; L-cysteine

sulfinic acid; (2S, 1'S, 2'R)-2-(carboxycyclopropyl)glycine; DL-TBOA; and closely related analogs thereof, and optionally, instructions for use.

19. A method as in claim 1, wherein said inhibitor demonstrates one or more of the following activities:

a) demonstrates the ability to block the reduction of electrical activity following anoxia;

b) demonstrates the ability to block the reduction of electrical activity following mechanical injury;

c) demonstrates the ability to decrease the ability of a Na+ dependent glutamate transporter to take up a neurotransmitter; and/or

d) demonstrates the ability to decrease the transport of D-aspartate efflux.

20. A kit for reducing the damaging effect of CNS white matter injury to mammalian CNS tissue comprising: a catheter and a formulation comprising is one or more compounds selected from the list comprising: L-trans-PDC; dihydrokainate; L-trans-2,4-pyrrolidine dicarboxylate; L-anti-endo-3,4-methanopyrrolidine dicarboxylate; an isomer of L-anti-endo-3,4-methanopyrrolidine dicarboxylate; DL-threo-beta-hydroxyaspartic acid; L-cysteic acid; L-cysteine sulfinic acid; (2S, 1'S, 2'R)-2-(carboxycyclopropyl)glycine; DL-TBOA; and closely related analogs thereof, and optionally, instructions for use.

FIGURE 1

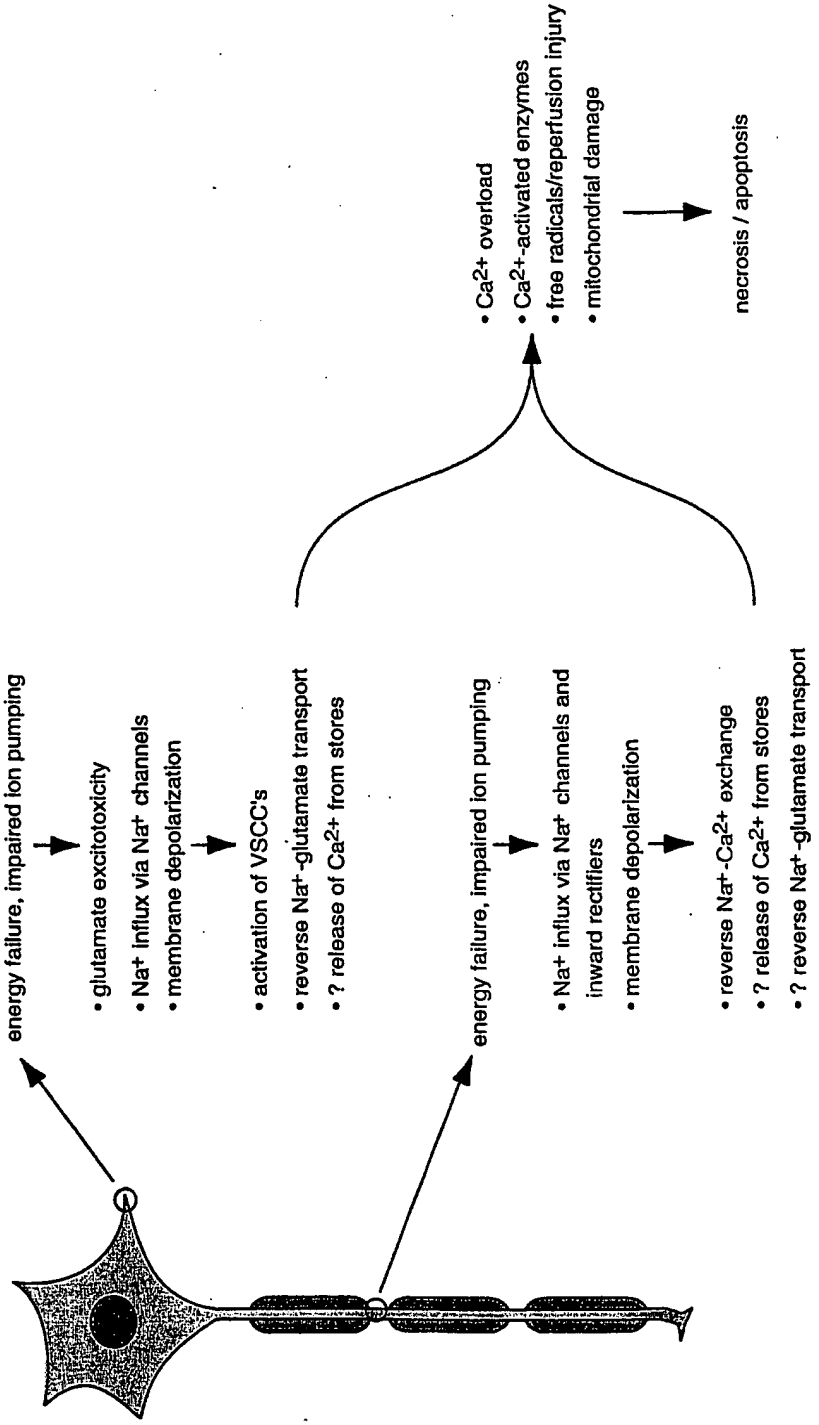

energy failure, impaired ion pumping
• glutamate excitotoxicity
• Na+ influx via Na+ channels
• membrane depolarization
• activation of VSCC's
• reverse Na+-glutamate transport
• ? release of Ca2+ from stores
• Ca2+ overload
• Ca2+-activated enzymes
• free radicals/reperfusion injury
• mitochondrial damage
necrosis / apoptosis
energy failure, impaired ion pumping
• Na+ influx via Na+ channels and inward rectifiers
• membrane depolarization
• reverse Na+-Ca2+ exchange
• ? release of Ca2+ from stores
• ? reverse Na+-glutamate transport

FIGURE 2

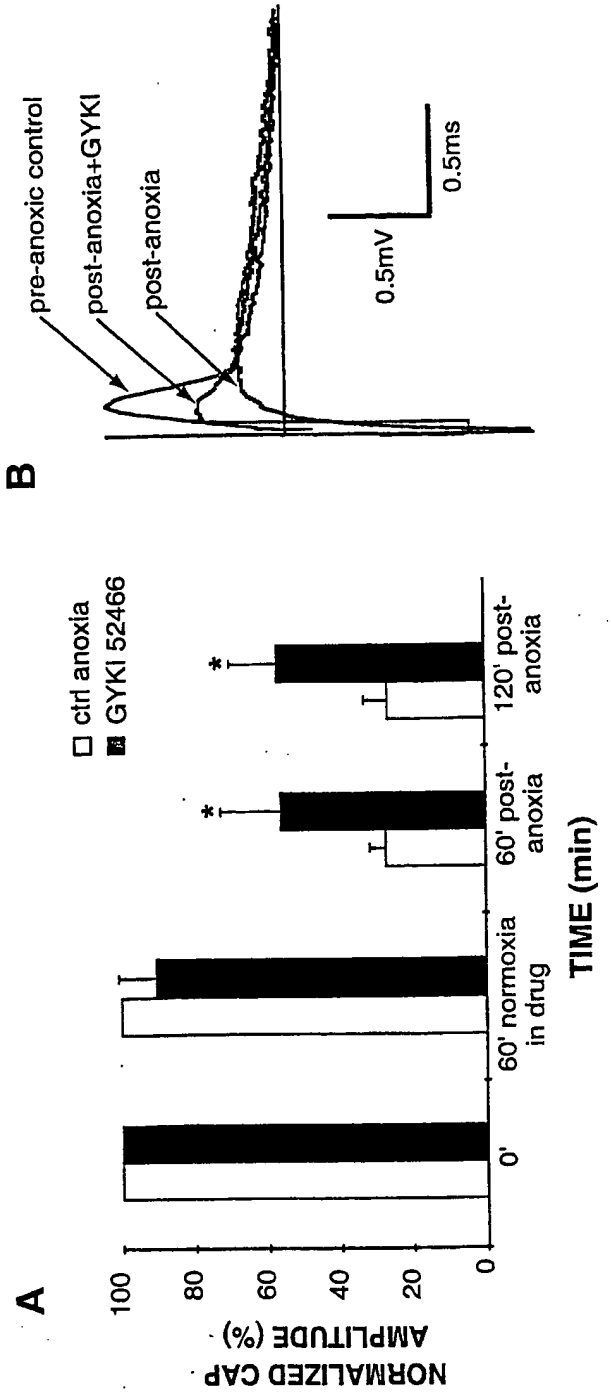

B
pre-anoxic control
post-anoxia+GYKI
post-anoxia
0.5mV
0.5ms
A
NORMALIZED CAP AMPLITUDE (%)
100
80
60
40
20
0
ctrl anoxia
GYKI 52466
*
*
0'
60' normoxia in drug
60' post-anoxia
120' post-anoxia
TIME (min)

FIGURE 3

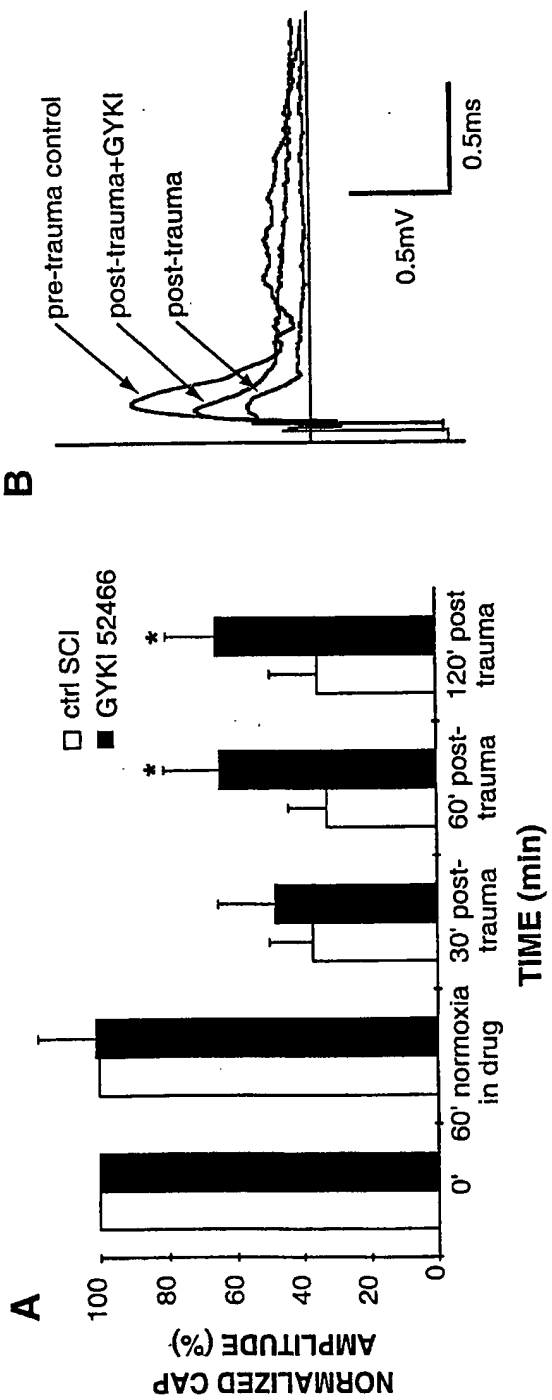

B
pre-trauma control
post-trauma+GYKI
post-trauma
0.5mV
0.5ms
A
NORMALIZED CAP AMPLITUDE (%)
100
80
60
40
20
0
□ ctrl SCI
■ GYKI 52466
*
*
0' 60' normoxia in drug
30' post-trauma
60' post-trauma
120' post trauma
TIME (min)

FIGURE 4

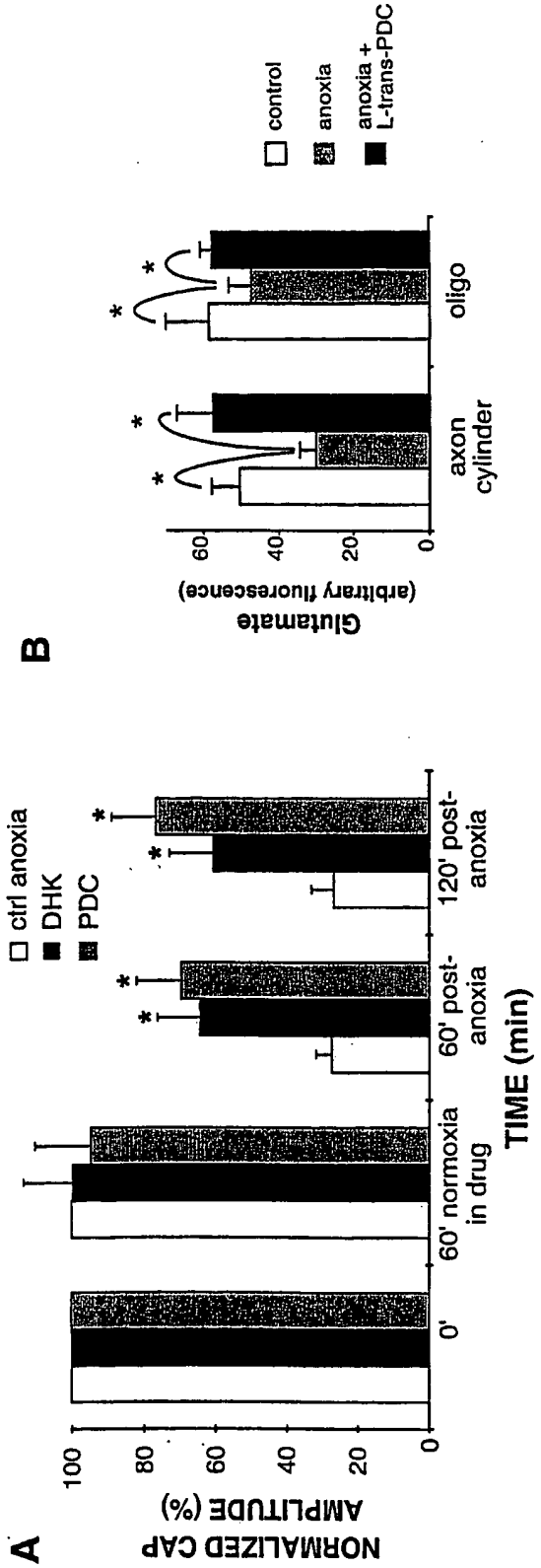
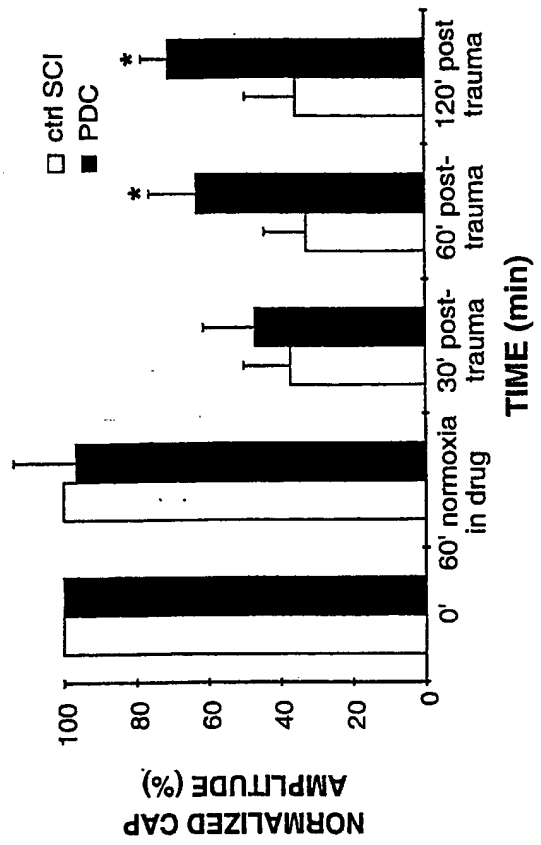

A
NORMALIZED CAP AMPLITUDE (%)
0
20
40
60
80
100
ctrl anoxia
DHK
PDC
0'
60' normoxia in drug
60' post-anoxia
120' post-anoxia
TIME (min)
B
Glutamate (arbitrary fluorescence)
0
20
40
60
control
anoxia
anoxia + L-trans-PDC
axon cylinder
oligo
*

FIGURE 5


ctrl SCI
PDC
*
*
0'
60' normoxia in drug
30' post-trauma
60' post-trauma
120' post trauma
TIME (min)
0
20
40
60
80
100
NORMALIZED CAP AMPLITUDE (%)